Fluorescence Lifetime Measurements

I. Introduction to time resolved fluorescence measurements
II. Types of measurements
III. Pulse lifetime measurements
IV. Instrument components
V. Single photon counting method (TCSPC)
VI. Deconvolution
VII. Other Corrections

I. Introduction to Time-resolved Fluorescence Measurements

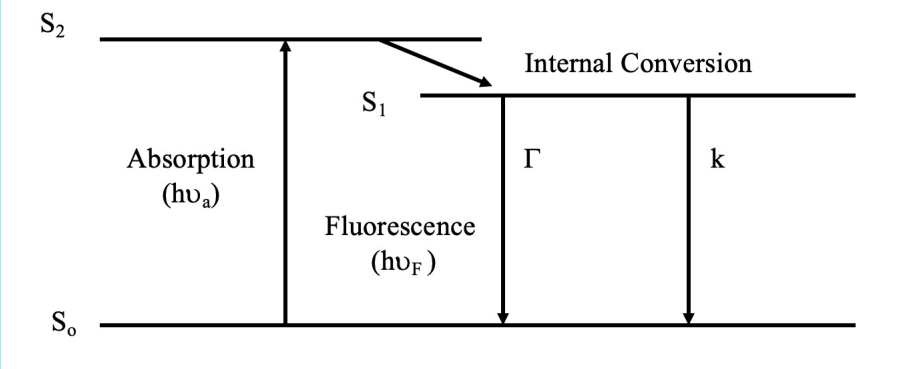
Lifetime

Lifetime of the excited state is the average time a molecule spends in the excited state before returning to ground state (~ 1 - 10 ns)

$$\tau = \frac{1}{\Gamma + K}$$

K - rate of radiationless decay [s^{-1}]; Γ - rate of radiative decay (fluorescence) [s^{-1}]

Both depopulate the excited state



I. Introduction to Time-resolved Fluorescence Measurements

- Fluorescence decay characteristics can tell us about the interactions of the fluorophore with its environment
- Fluorescence lifetimes are typically ~ 1-10 nano seconds; difficult to measure, requiring high speed electronic devices
- Delayed fluorescence (and phosphorescence) lifetimes are much longer ~ 1-100 micro seconds

I. Introduction to Time-resolved Fluorescence Measurements

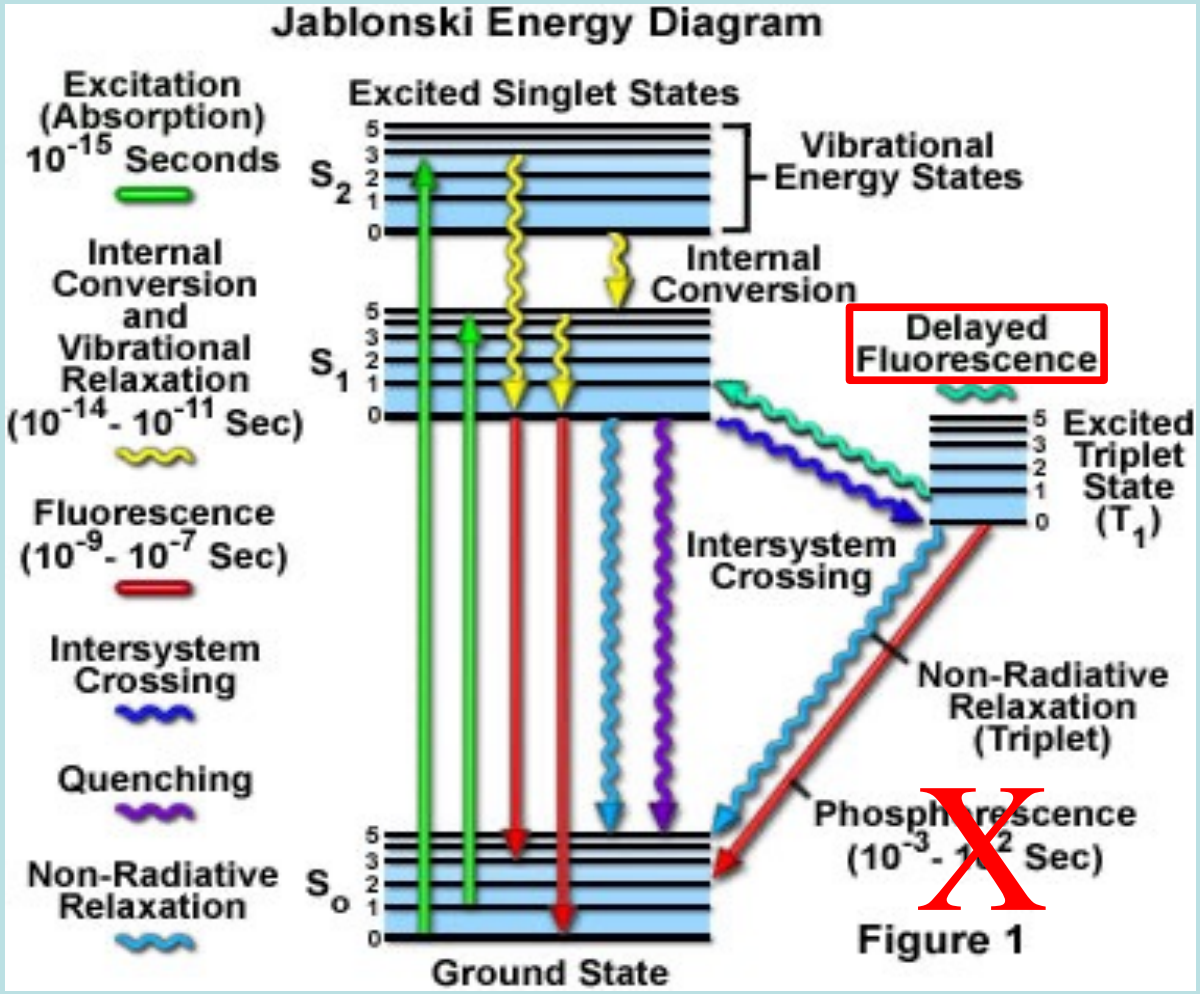
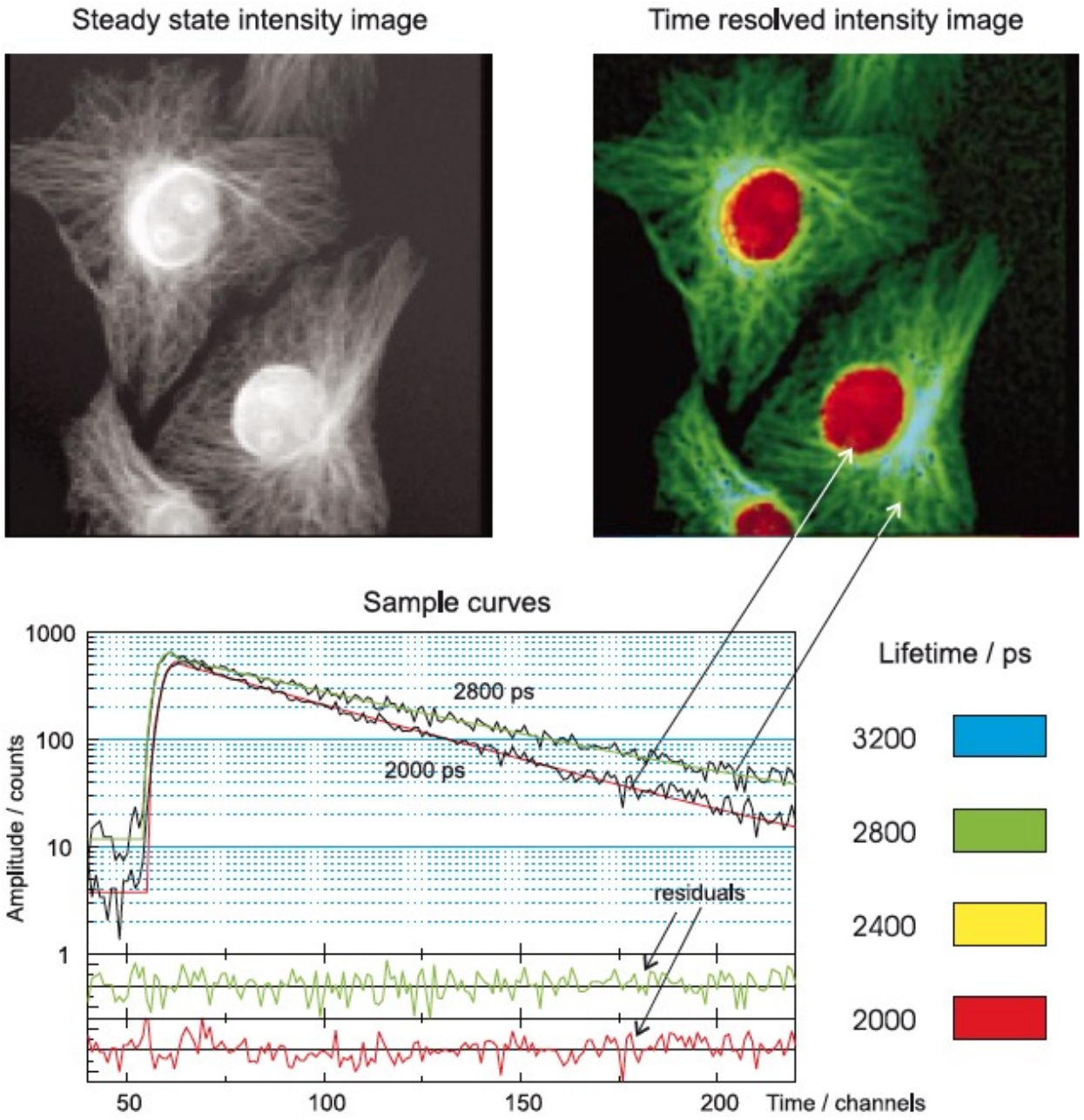


Figure 1



The fluorescence lifetime provides additional information for:

- Contrast enhancement

- Characterization

- Quantification

I. Introduction to Time-resolved Fluorescence Measurements

Characteristics of life time

Consists to looks at how excited states depopulate over time

- Can reflect properties of environment
- Does not depends on the concentration
- Can reflect molecular dynamics of molecule (**Quenching**)

I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence Quenching

Quenching refers to any process that causes a reduction in the quantum yield of a given fluorescence process.

Static quenching is due to the formation of a ground state non-fluorescing complex (i.e. before excitation occurs) between the fluorescent molecule and the quencher with formation constant K_c, described by:

$$I_o/I = 1 + K_c[Q]$$

where I_o is the fluorescence intensity in the absence of quencher, I is the intensity in the presence of quencher at concentration [Q].

The observed lifetime does not appear in this equation and is independent of quencher concentration in static quenching.

I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence Quenching

Static quenching:

Frequently induced by dye aggregation which are often due to hydrophobic effects—the dye molecules stack together to minimize contact with water.

- Planar aromatic dyes that are matched for association through hydrophobic forces can enhance static quenching.

- High temperatures and addition of surfactants tend to disrupt ground state complex formation.

I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence Quenching

Collisional (dynamic) quenching, encounters between excited fluorophore and quencher. It is described by the Stern-Volmer Equation:

$$I_0/I = \tau_0/\tau = 1 + k_q[Q]\tau_0$$

where I_0 is the fluorescence intensity in the absence of quencher, I is the intensity in the presence of quencher at concentration [Q] (idem for τ), k_q is the rate of collisional quenching, and τ_0 is the observed lifetime in the absence of quencher. **Collisional quenching is clearly observed when there is a linear increase of $1/\tau$ with increasing quencher concentration.**

I. Introduction to Time-resolved Fluorescence Measurements

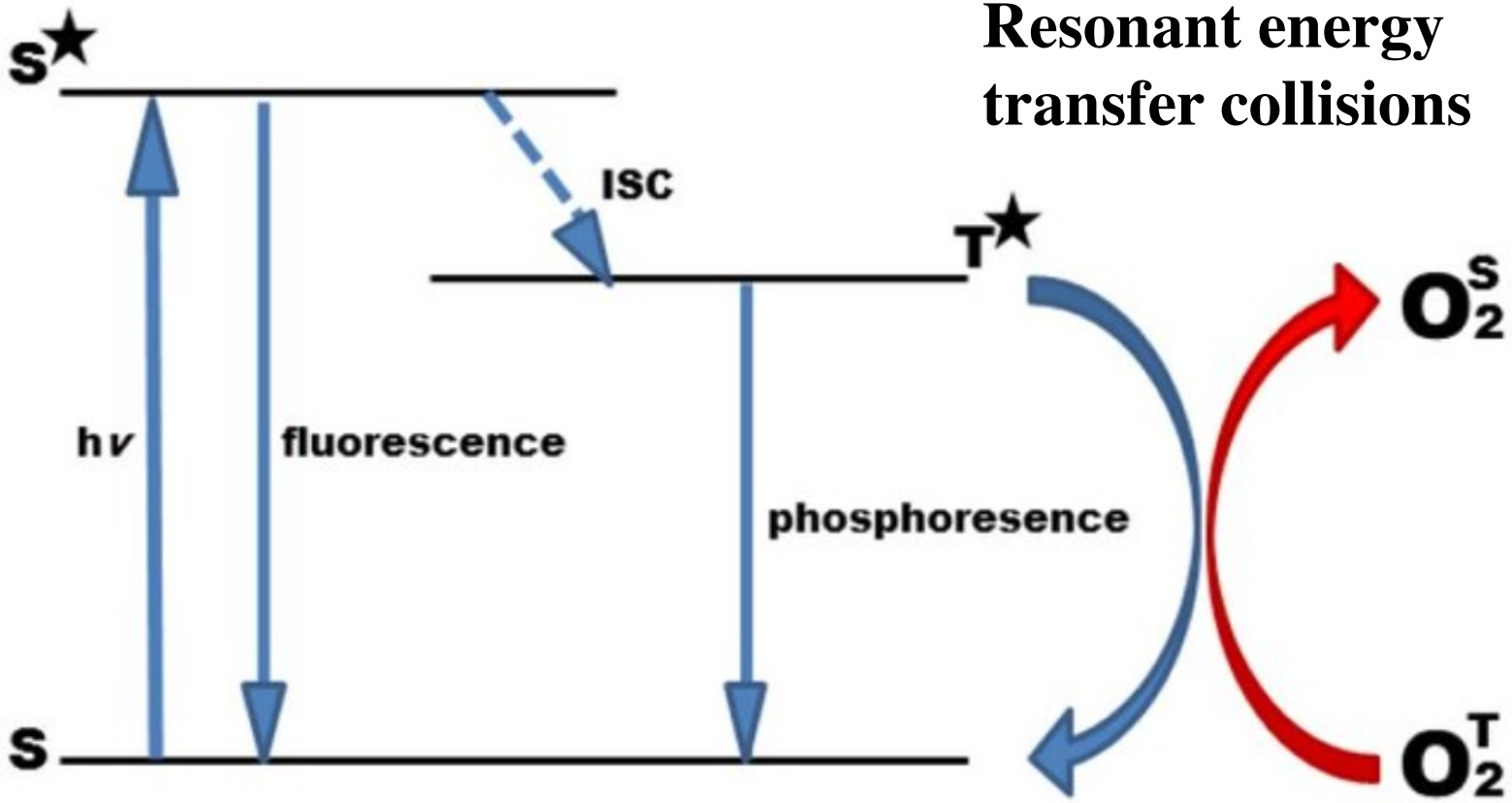
Fluorescence Quenching

Mechanism of collisional quenching

- The quencher must diffuse to the fluorophore during the lifetime of its excited state.

- Upon contact the fluorophore returns to the ground state, without emission of a photon

I. Introduction to Time-resolved Fluorescence Measurements



I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence Quenching

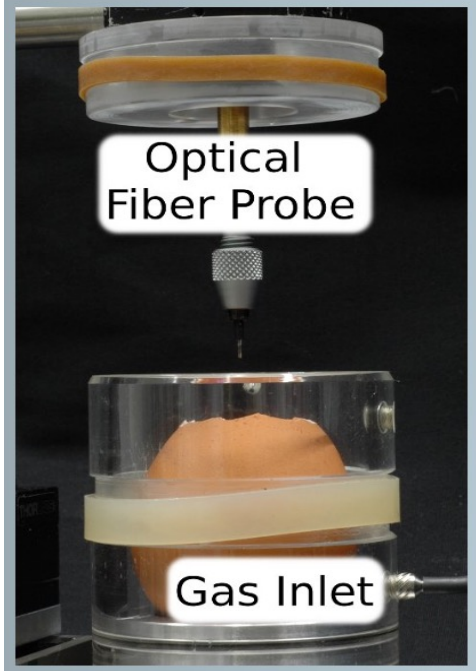
Collisional quenchers

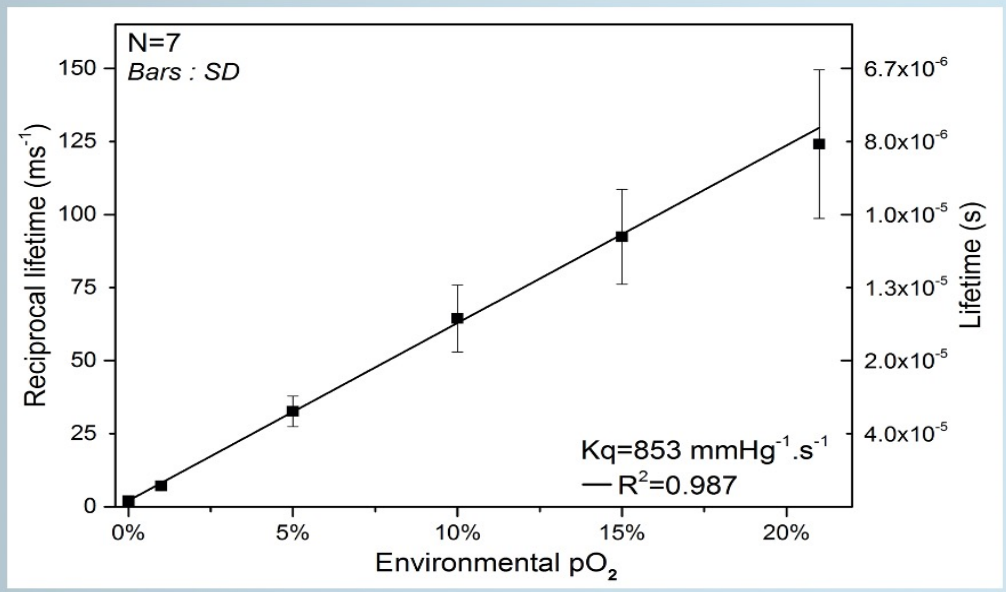
- Molecular oxygen O_2 – one of the best known collisional quenchers (likely mechanism – inter system crossing to triplet state)
- Other collisional quenchers include: Xenon, hydrogen peroxide, acrylamide, nitrous oxide, halogen containing substances, etc

I. Introduction to Time-resolved Fluorescence Measurements

Evolution of the PpIX delayed fluorescence lifetime for different pO_2 in vivo on the chick's embryo chorioallantoic membrane (CAM)

pO_2 is changed by a local gas flow with defined oxygen concentration (0, 2, 5, 10, 15 and 21 % O_2 in N_2)



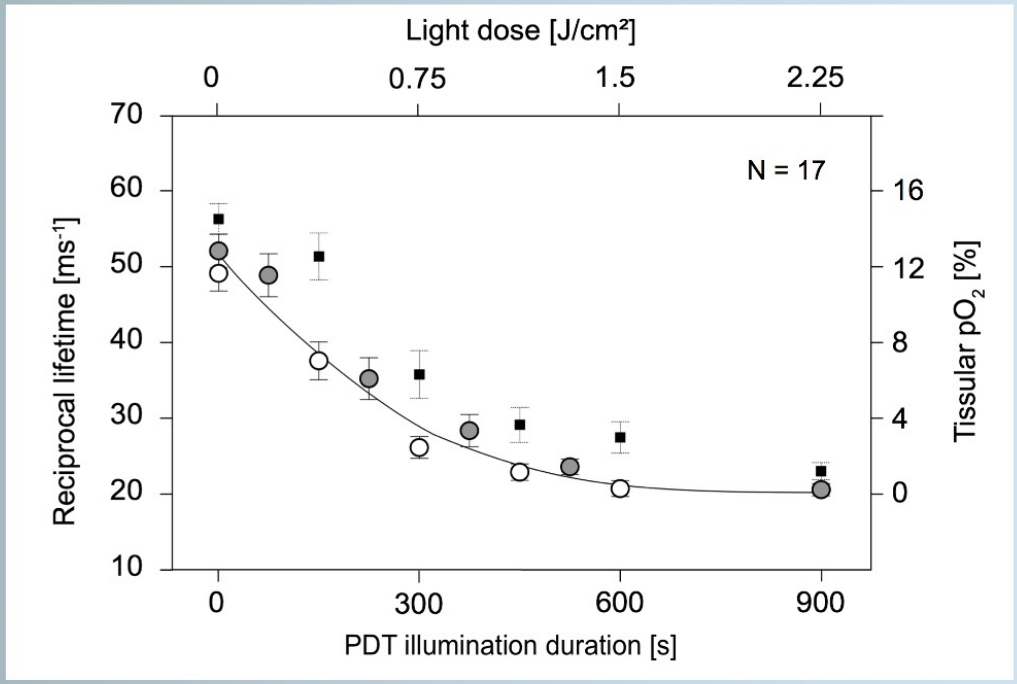


→ Linear correlation between pO_2 and the reciprocal lifetime of PpIX triplet state

Piffaretti et al., JBO, 2012

I. Introduction to Time-resolved Fluorescence Measurements

Tissular oxygen depletion during photodynamic therapy of the CAM



Bars: SEM

Different symbols: different measurement series

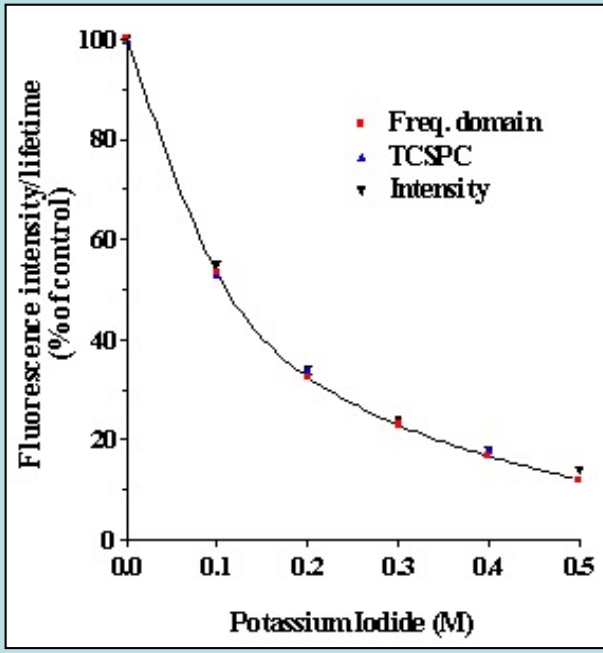
- The pO_2 decreases monotonically as the PDT illumination increase.
- A fraction of J/cm^2 @ 405 nm deplets oxygen!
- This is a promizing approach to monitor the light dose during PDT!

Piffaretti et al., JBO, 2012

I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence collisional Quenching

Quenching of fluorescein by potassium iodide (KI)



[KI] molar	Freq. domain (ns)	TCSPC (ns)	Intensity
0	3.99	3.99	7.87
0.1	2.13	2.20	4.32
0.2	1.30	1.34	2.67
0.3	0.92	nd	1.87
0.4	0.67	0.72	1.41
0.5	0.47	nd	1.08

$$I_0 / I = \tau_0 / \tau$$

I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence Quenching

Selectivity of quenching

- Not all fluorophores are quenched by all of the substances listed previously (in slide 12).
- There is selective quenching of a given fluorophore.
- This selectivity does depend on the structure of individual molecules – detailed analysis is complex.

I. Introduction to Time-resolved Fluorescence Measurements

Selectivity of quenching

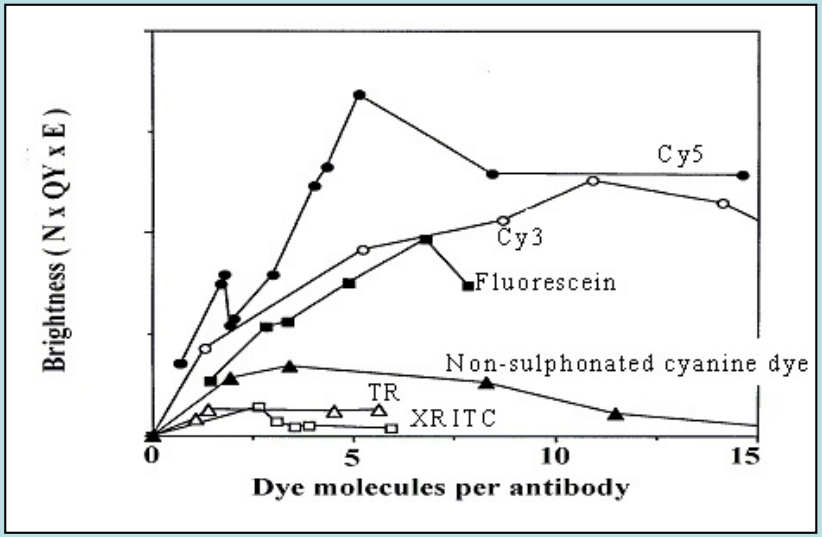
Table 1: Environmental sensitive dyes for sensing applications

Dye	Application
Nitrobenzodiazole (NBD), Laurdan, di-ANEPPDHQ, Prodan	Investigations of membrane structure and composition
MQAE, lucigenin-QDot nanosensor, Clomeleon	Cl^--concentration measurements in tissue
BCECF, Carboxyl- (c) SNAFL1, C-SNAFL2, fluorescein, C-fluorescein	pH measurements in living cells/tissues
Dentrimer compounds	Explosive sensing
Metall ligand complexes	Oxygen sensing
NADH, 2FPBA/Alirazin red	Glucose sensing
8-(alkoxy)-quinoline based probes	Protein content measurements
Rhodamine B, quantum dots	Temperature measurements
Calmodulin, Mermaid, GcaMP2, di-8-ANEPPS, TX-XL, Calcium Green	Ca^{2+} imaging

I. Introduction to Time-resolved Fluorescence Measurements

Fluorescence Quenching

Self quenching: in some cases as concentration of fluorophores increases so the overall fluorescence intensity decreases due the phenomenon of "self quenching". Many fluorescent molecules exhibit self-quenching where the presence of large concentrations of dyes will significantly impact on the quantum yield. Different fluorophores quench variably under certain conditions.



A protein has been labelled with varying quantities of fluorescent dyes

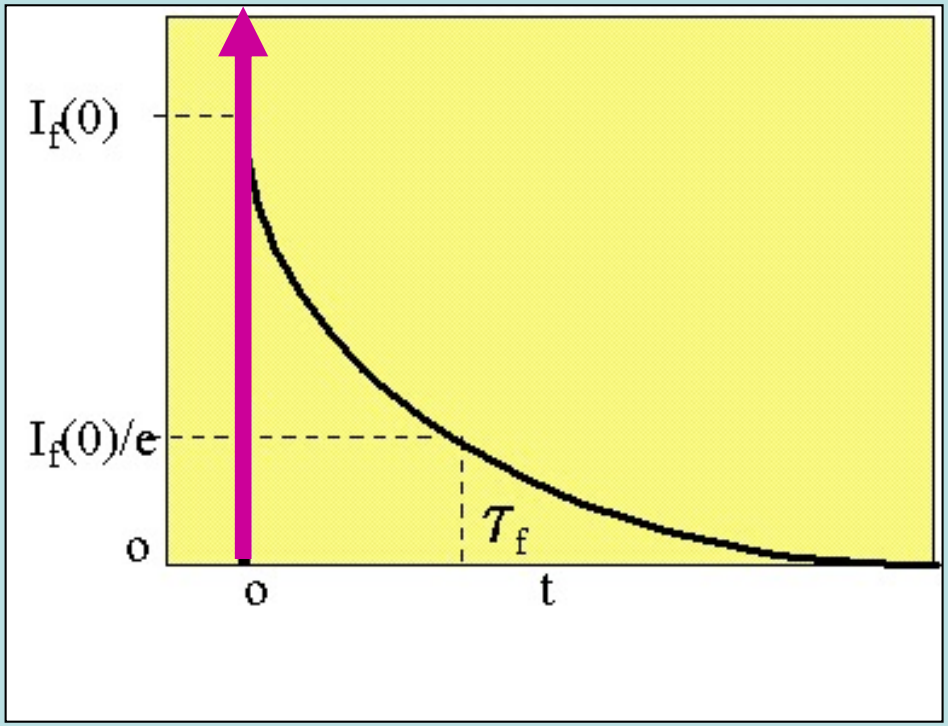
II. Types of Measurements

- **Time domain (pulse) method**
  1. Sample is excited with short pulse of light
  2. Time dependent decay of fluorescence intensity is measured and used to calculate lifetime

- **Frequency domain (phase) method**
  1. Sample is excited with sinusiodally modulated light
  2. The phase shift and demodulation is used to calculate the lifetime

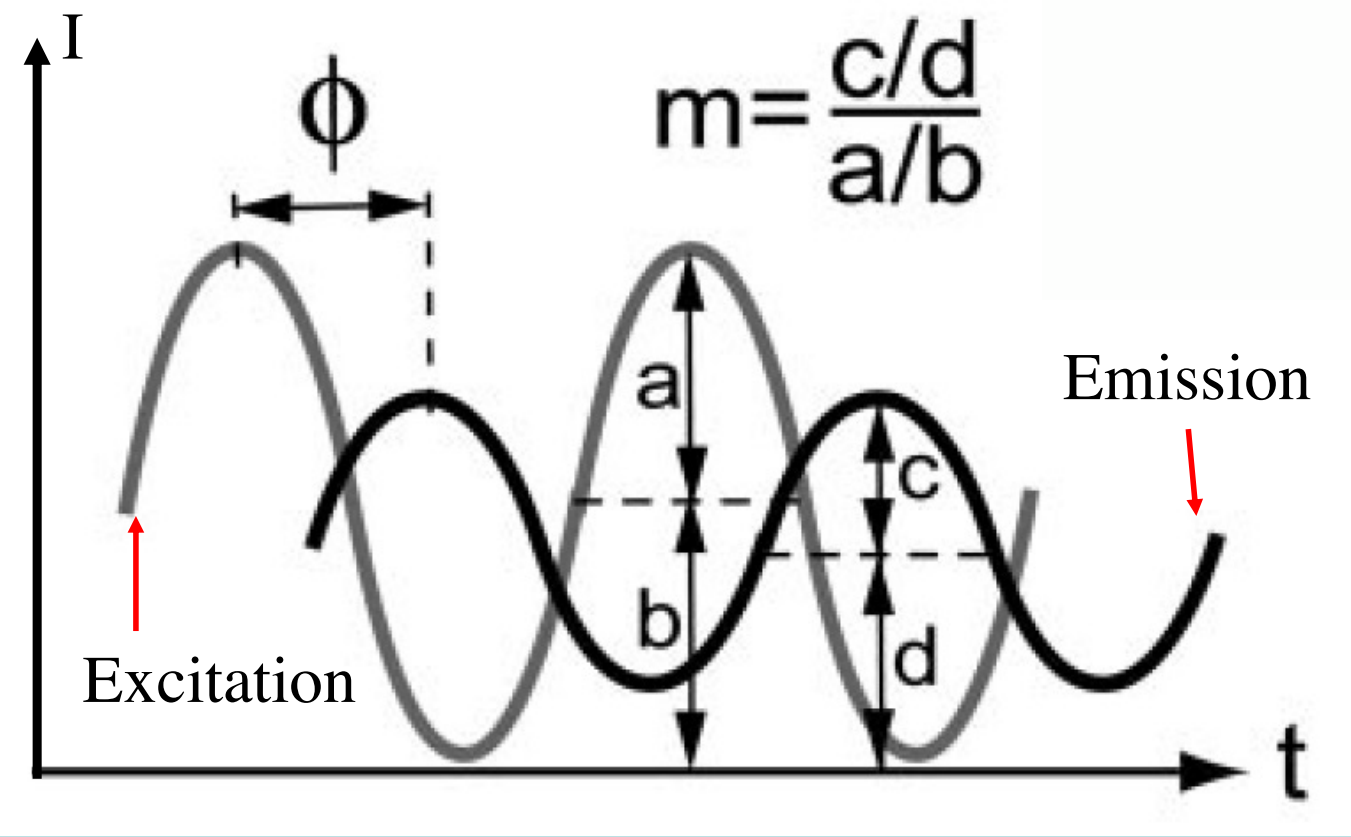
II. Types of Measurements

Time domain: Pulsed excitation



II. Types of Measurements

Frequency domain method



$$\tau_\phi = \frac{1}{2\pi f}\tan(-\phi)$$

$$\tau_m = \frac{1|}{2\pi f}\sqrt{m^{-2}-1}$$

III. Pulse lifetime measurements

Lifetime measurements with pulse method

- Fluorophore is excited with infinitely short pulse of light (ideal case)
- This results in an initial population of fluorophores in excited state N_0.
- The rate of decay of the initially excited population can be described analytically.

III. Pulse lifetime measurements

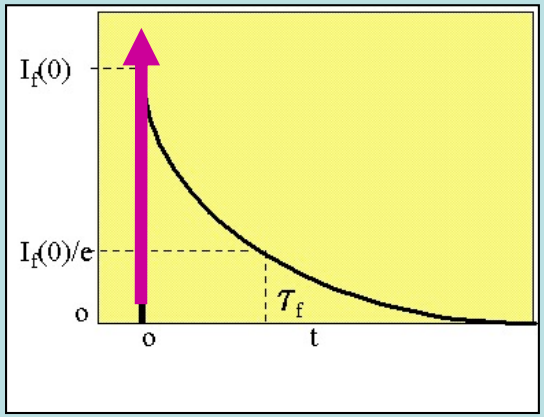
Lifetime measurements with pulse method

- The fluorescence intensity I(t) (energy) is proportional to the excited state population
- I(t) will decay exponentially in the simplest case.
- The fluorescence lifetime is generally equated with the time required for the intensity to decay to 1/e of its initial value (monoexponential decay)

III. Pulse Lifetime Measurements

For a single fluorophore, which decays mono-exponentially :

$$I(t) = \Gamma N_o e^{-t/\tau} = I_o e^{-t/\tau}$$



where,

I_o is the fluorescence intensity (power) at time, t equal to 0.

III. Pulse lifetime measurements

Multi-exponential decays are frequently observed:

- Mixture of non-interacting fluorophores
- One fluorophore in different environments
- Energy transfer or solvent relaxation …

Fluorescence decay is frequently non-monoexponential and can be described by the sum of multi exponential decays (not always!).

III. Pulse Lifetime Measurements

For decays not described by a single exponential, decay fit to a sum of exponentials:

$$I(t) = \sum_i \alpha_i e^{-t/\tau_i}$$

where,

α_i represents the fractional contribution of the component with lifetime, τ_i.

!! Decays are sometimes not mono-exponential and neither multi-exponential (second order effects due to transitions between multiple excited states) !!

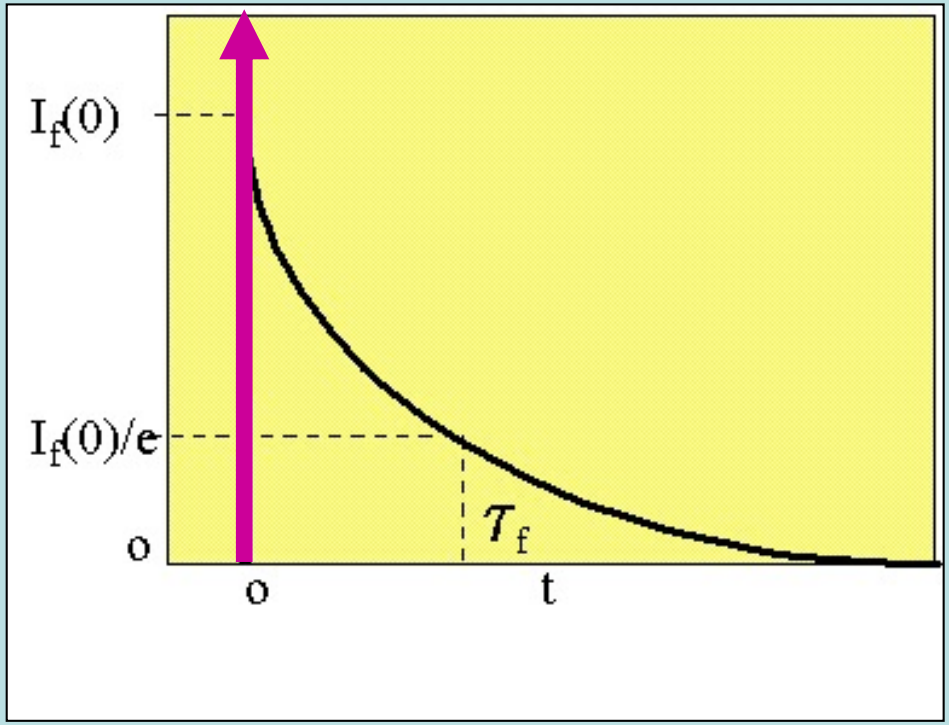
IV. Instrument Considerations

Ideal requirements (time-domain methods)

- Measurements of fluorescence lifetimes by the pulse method requires the measurements of the time resolved decay of fluorescence intensity
- Infinitely narrow excitation pulse (δ pulse excitation)
- Detector with high gain and sub pico second response time

IV. Instrument Considerations

Ideal requirements



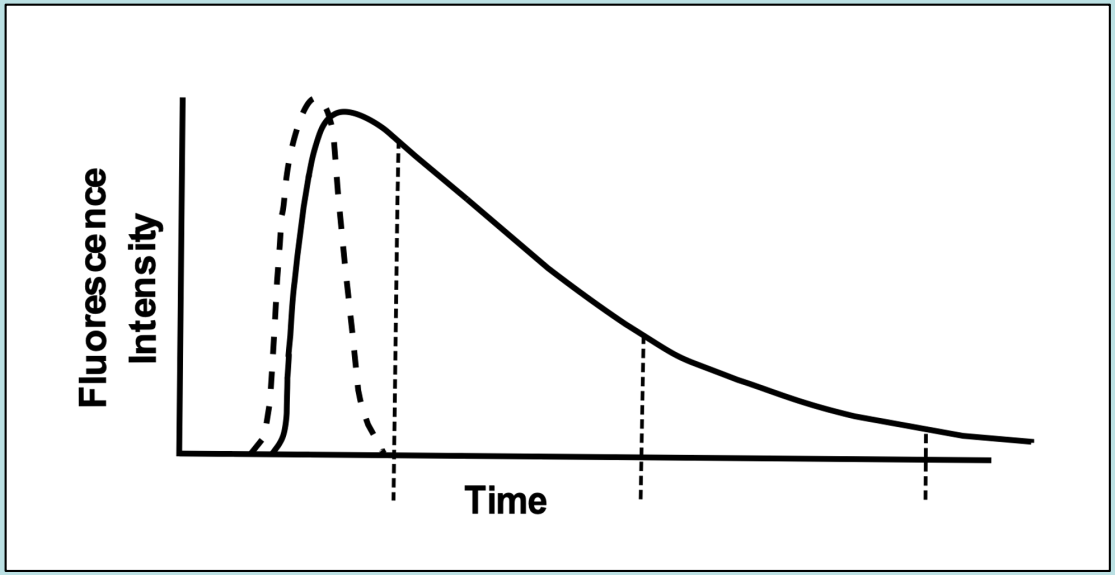
IV. Instrument Considerations

Realistic situation

- Only several available light sources yield pulses of sub-nano second duration
- Detectors cannot measure entire time resolved fluorescence decay with one excitation pulse

IV. Instrument Considerations

Realistic situation



V. Time-correlated Single Photon Counting (TCSPC)

1. Principles
2. Methodology
3. Components of instrumentation
4. Temporal distribution

V. Time-Correlated Single Photon Counting

Why Time-Correlated Single-Photon Counting?

After a fluorescent molecule is excited by a short laser pulse, it will emit a photon at some random time later, usually in the nanosecond region. In the limit of a very large number of molecules emitting photons in this way, one commonly observes an *exponential* decay of the intensity with time. If the intensity is measured in a single experiment, such as following the use of an intense laser pulse to excite many molecules, the amount of signal detected and the observed time profile depend on the response of a detector to the light intensity. Detectors are limited, since:

(a) Their response is often linear over only a small range of intensity. That is, the intensity falling onto the detector is not fully reproduced in the detector output.
(b) Their response times (bandwidth) are often too long.

Many experiments require fluorescence intensities to be measured as a function of time with much greater precision than available from "standard" time domain experiments.

V. Single Photon Counting

1. Principles

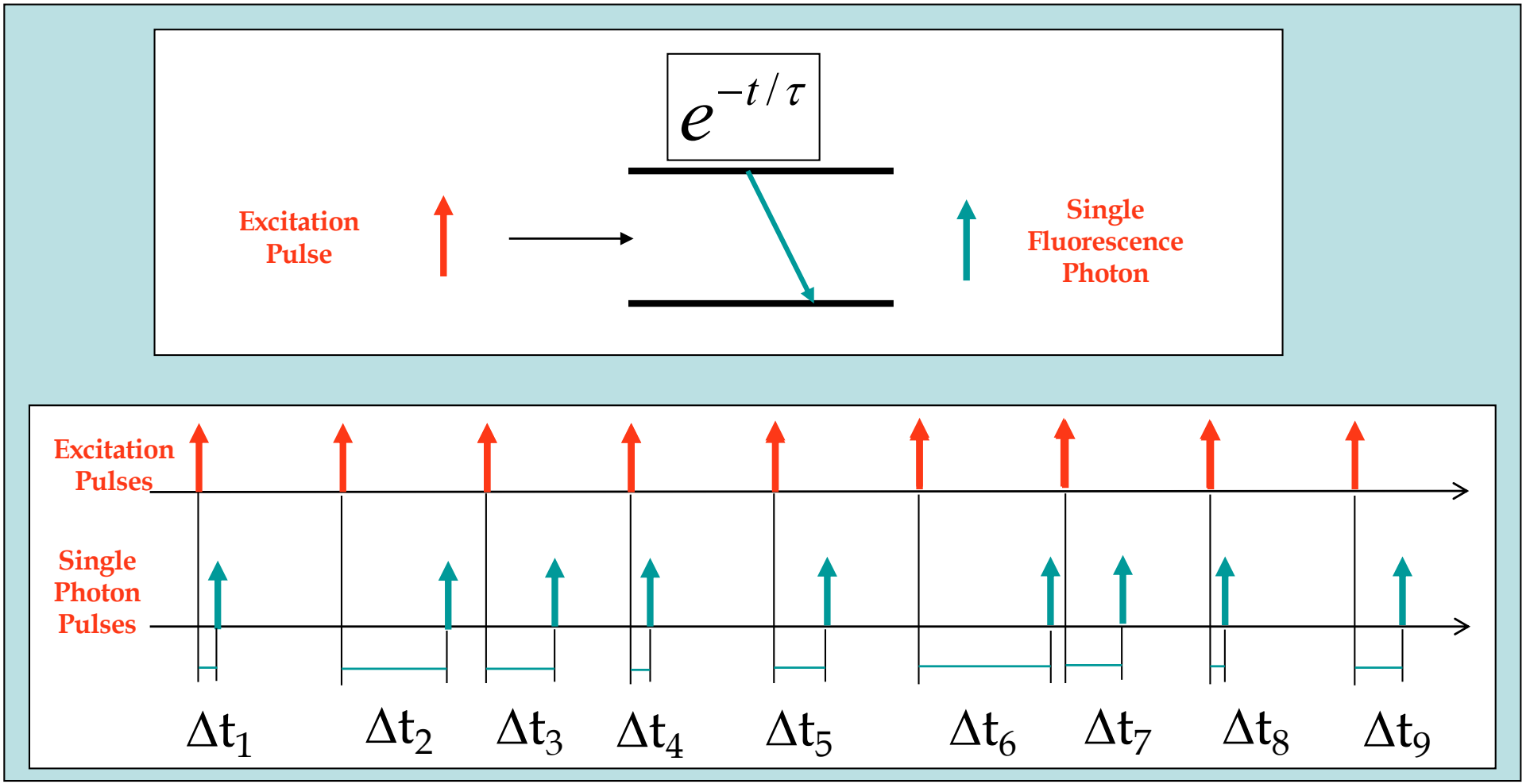
- Fluorescence emission is a random event and follows a probability distribution determined by the lifetimes (PDF: exponential(s))
- If a single fluorescent photon is detected, the probability of emission can occur at any time within the distribution of lifetimes
- If multiple fluorescent photons are detected, their distribution represents the lifetime of the fluorophore, or the time resolved fluorescence decay

V. Single Photon Counting

2. Methodology

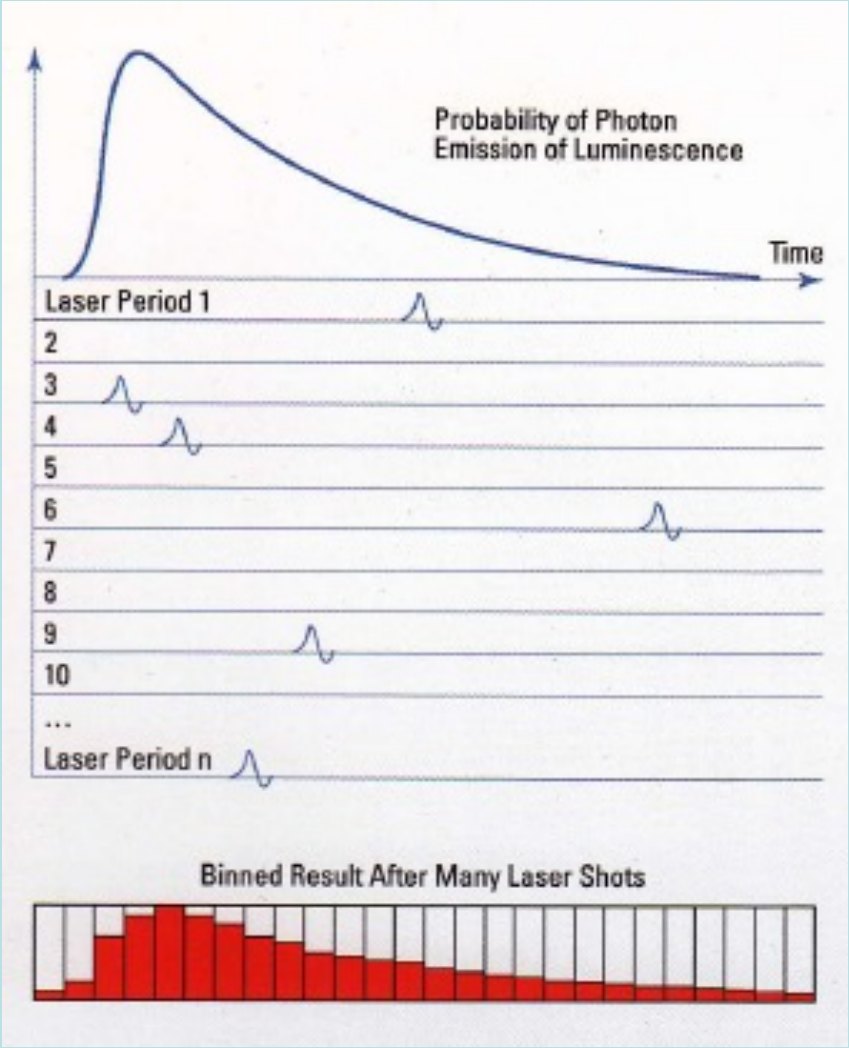
- Sample is excited with a pulsed light source
- Sample emits fluorescence
- Time interval between excitation short pulse and the first pulse at the detector is measured

V. Single Photon Counting



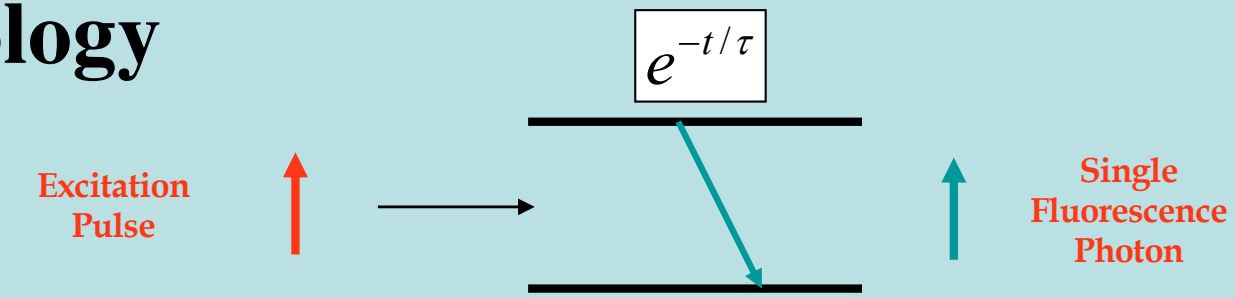
V. Single Photon Counting

2. Methodology

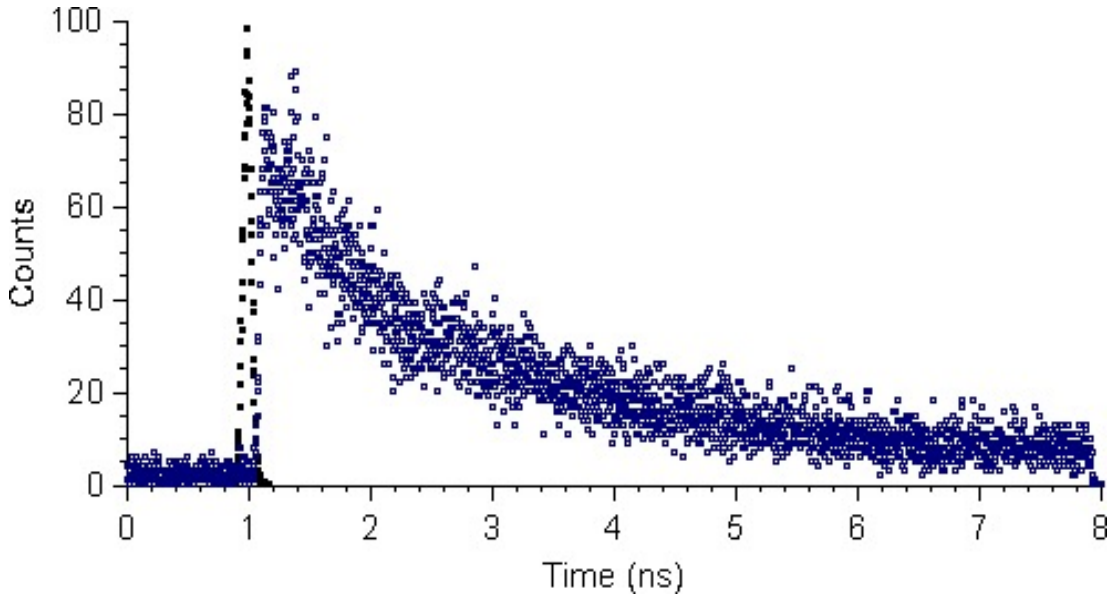


V. Single Photon Counting

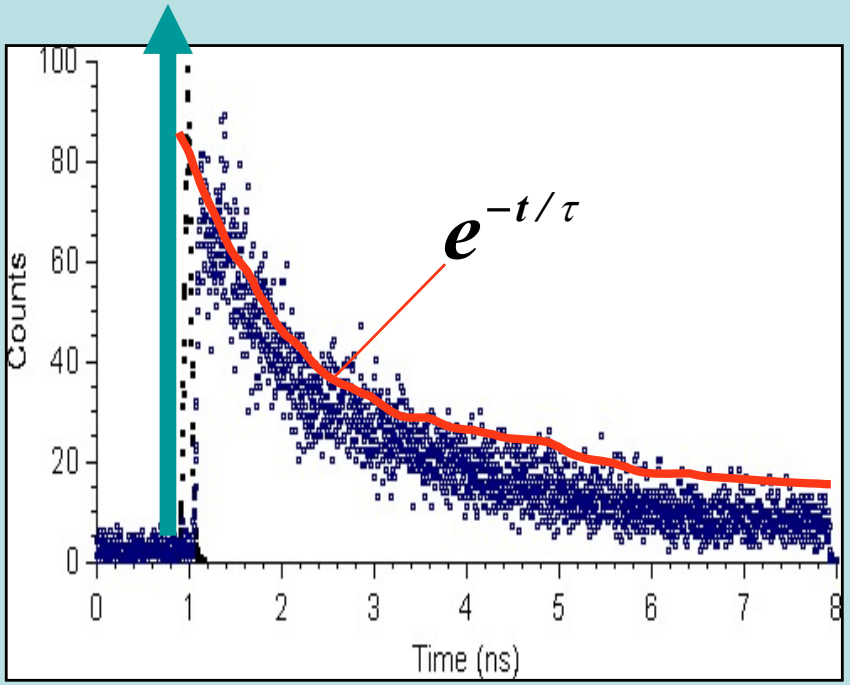
2. Methodology

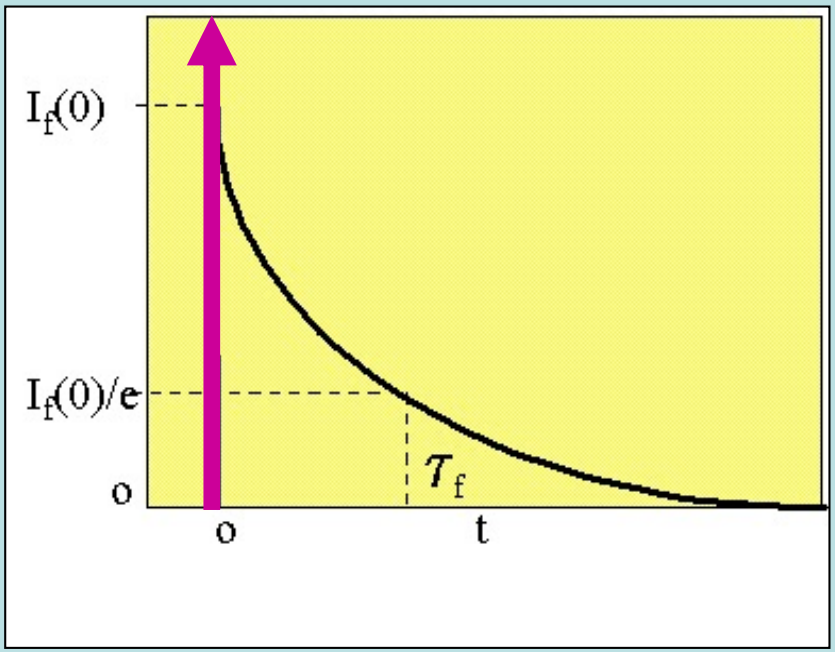


~ $10^{6\text{-}7}$ photons counted



V. Time-Correlated Single Photon Counting





V. Single Photon Counting

3. Components of instrumentation

- **Light sources**
  - Observed fluorescence decay must be corrected for the width of the light pulse via deconvolution
  - Permits 1 ns lifetime measurements with light pulse width of 2 ns
- **Detector**
  - Use single photon counting method to measure fluorescence decay (sensitive, low noise)
  - Time to amplitude converter (TAC) measures time between pulse and arrival of first photon for about 10^6 - 10^7 excitation pulses (reflects time resolved decay)

V. Single Photon Counting

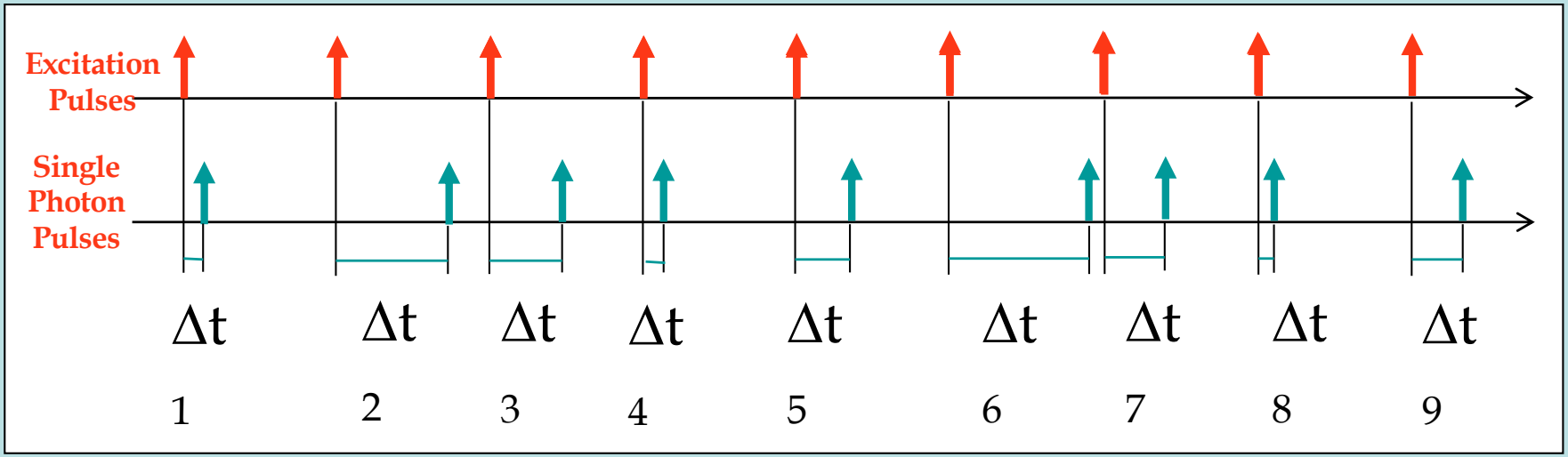
3a. Light sources

- **Gated lamps (only used for specific applications)**
  - Gated lamps operated with a thyratron (gas-filled tube used as a high-power electrical switch)
  - Typical lamps have pulse widths of 2 ns and low but long intensity tails
- **Lasers (mostly diode lasers)**
  - Pulsed lasers
  - Short pulses (pico second range)
  - High repetition rate (megahertz range: Typ. 80 MHz)

V. Single Photon Counting

3b. Detection

- **Single photon counting detectors**
  - Photomultiplier tubes (**PMT**; detection of single anode pulses)
  - Single Photon Avalanche Diodes (**SPAD**)
  - One photon or less is observed for every 20 excitation pulses (to minimize bias due to overlaps).



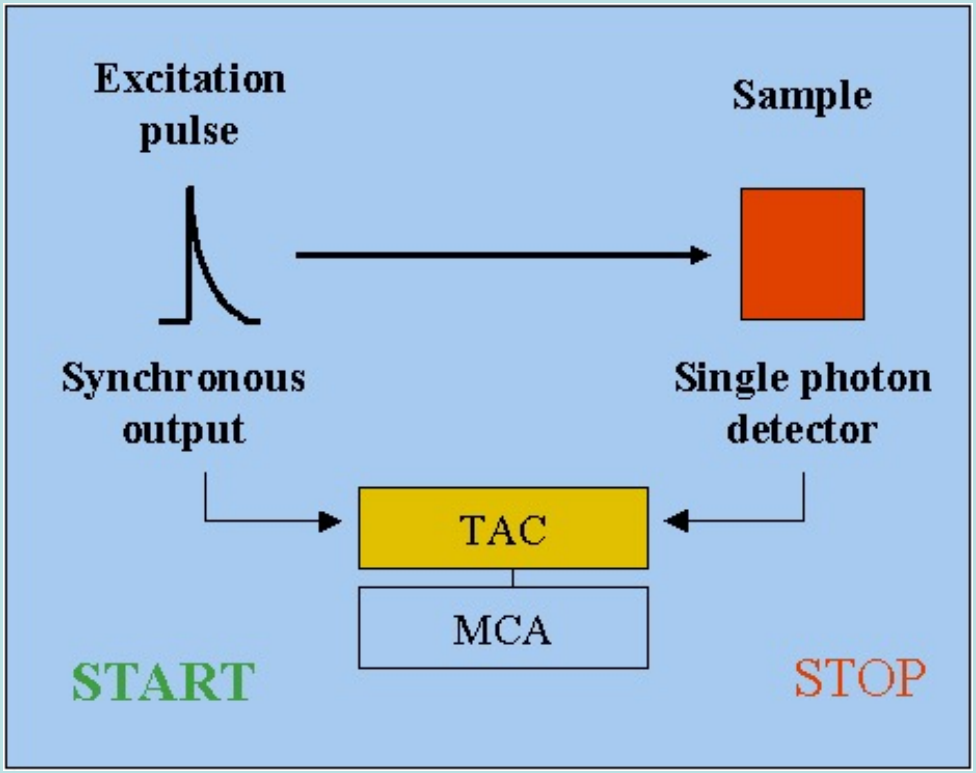
V. Single Photon Counting

3c. Detection and signal analysis:

- **Time interval measurement**
  - Time-to-amplitude converters (TAC)
  - Light pulse starts TAC; detector pulse stops TAC
  - Generates voltage which is proportional to the arrival time of photon
  - If no photon pulse, the TAC resets to zero
  - Detected pulses are recorded in bins of a multichannel pulse height analyzer (MCPHA or MCA)

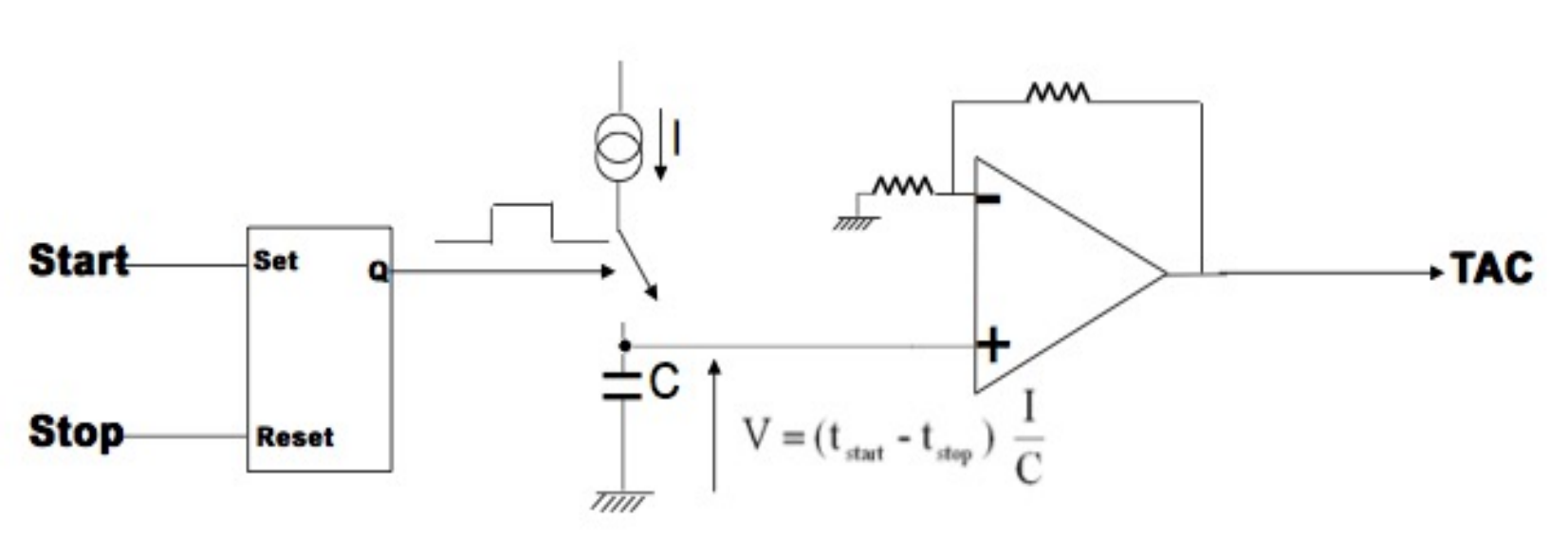
V. Single Photon Counting

3d. TCSPC: Instrumental aspects

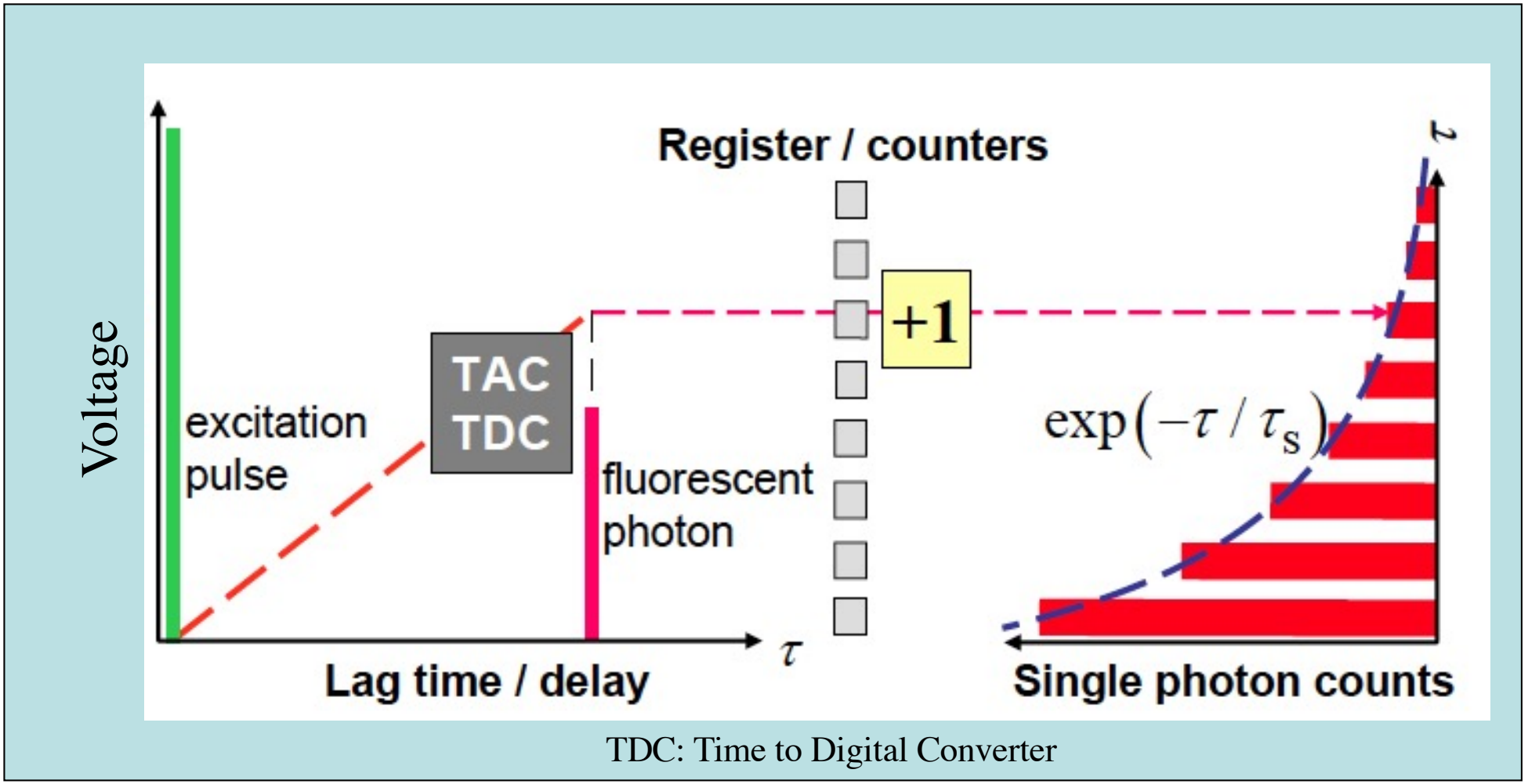


V. Single Photon Counting

3d. TCSPC: Instrumental aspects



V. Single Photon Counting



TDC: Time to Digital Converter

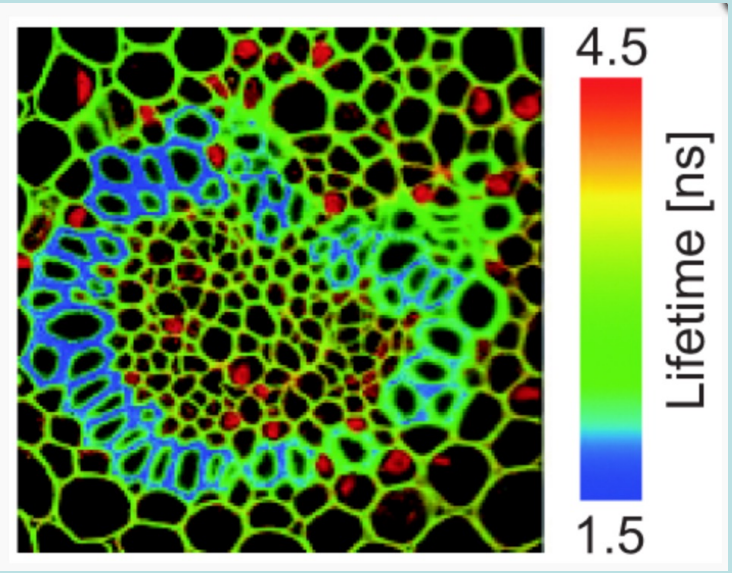
V. Single Photon Counting

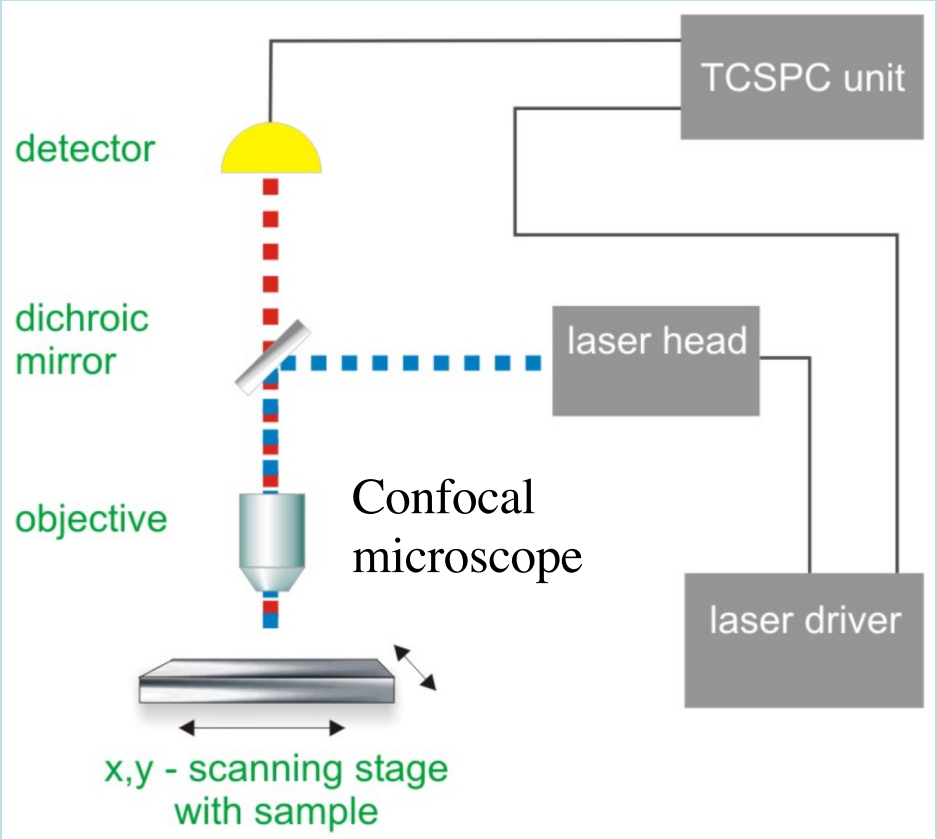
4a. Temporal distribution

- Procedure repeated at about 80 MHz until a large number of photon pulses are detected (10^6 - 10^8)
- MCPHA data (distribution of voltages) is plotted as a histrogram
- Data represents time resolved fluorescence decay

V. Single Photon Counting

- TCSPC Imaging





Autofluorescence lifetime image:
Unstained lime tree sample excited with a fs-pulsed laser at 1030 nm. The FLIM image upon three photon excitation shows strong contrast in the different morphological parts of the sample.

VI. Deconvolution

1. Objective
2. Accounting for finite width light pulse
3. Convolution of lamp pulse and fluorescence decay curve
4. Least squares method

VI. Deconvolution

1. Objective

- Yield time-resolved fluorescence decay for an infinitely sharp excitation pulse – impulse response function – F(t).

Free parameters of the fit: fluorescence lifetime, τ_i; Amplitude, α_i.

VI. Deconvolution

2. Analysis

- Need to deconvolve effect of finite width light pulse to get the fluorescence impulse response function F(t)
- The convolution of the light pulse response with the impulse response of the sample is given by:

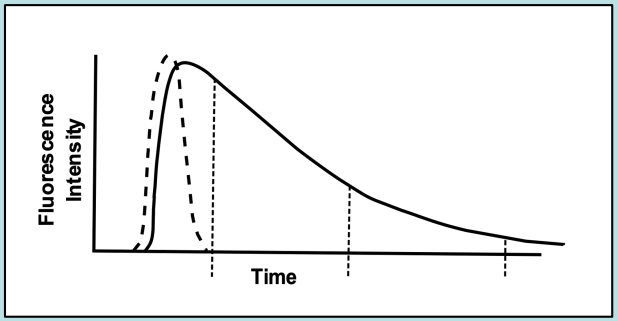
$$R(t) = \int_0^t L(t')F(t-t')dt' \qquad t > t'$$

where,

L(t') is the light pulse

F(t) is the impulse response function

t' is the time of excitation



VI. Deconvolution

3. When to apply a deconvolution

- Useful when light pulse widths are comparable to fluorescence decay times
- Need to measure the lamp pulse in a separate experiment i.e with a non-fluorescent solution that scatters light

VI. Deconvolution

4. Deconvolution methods

- Least squares method
  - Most reliable results
  - Widely applied to single photon counting data
  - Commercially available software

VI. Deconvolution

4a. Least squares method (iterative reconvolution)

- The goal is to obtain the parameters α_i and τ_i which provide the best match between the data $R(t)$ and the calculated decay $R_c(t)$ using assumed parameters values.

$$\chi^2 = \sum_{i=1}^{n} \frac{1}{R(t)} \left[R(t) - R_c(t) \right]^2$$

- This is accomplished by minimizing the goodness-of-fit parameter χ^2

Joseph R. Lakowicz: Principles of Fluorescence Spectroscopy, Springer, 2nd edition, 1999

VI. Deconvolution

4b. Least squares method (iterative reconvolution)

- Can improve the fit by increasing the number of exponential decays
- R(t) is never a single exponential decay function, unless the lamp pulse is infinitely short
- Need residuals to see the error of the fit

  (The residuals from a fitted model are defined as the differences between the response data and the fit to the response data at each predictor value)

VI. Deconvolution

4c. Least squares method (Residuals)

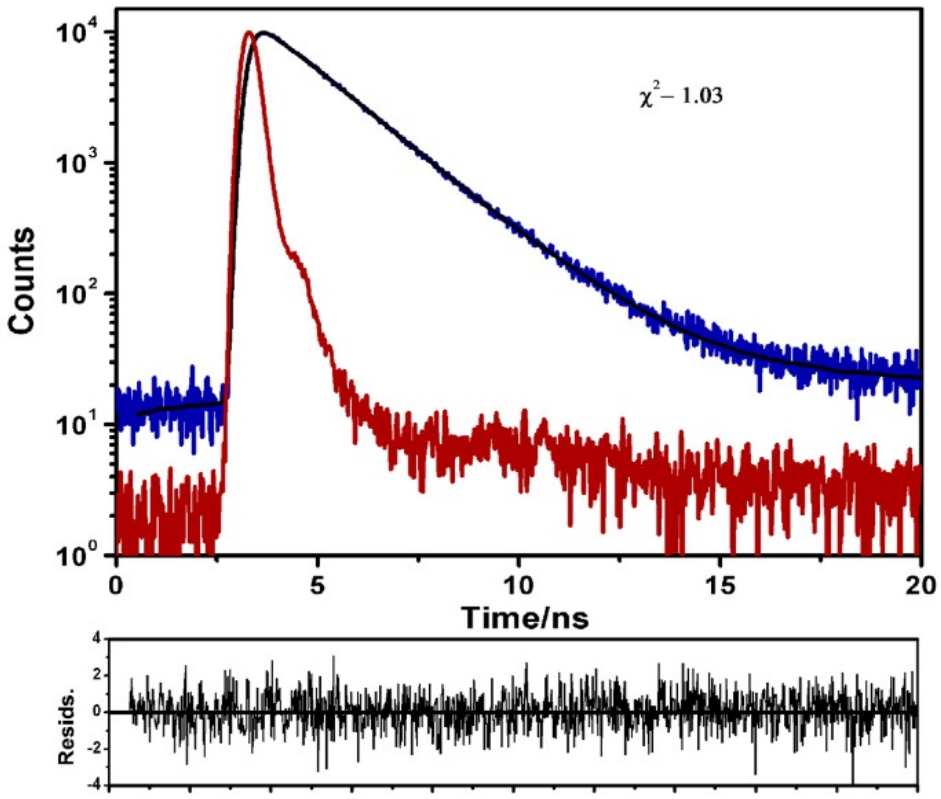


Fig. 3. Photoluminescence decay curve (λ_{em} – 516 nm) of freshly prepared SDS capped CdS quantum dot measured at room temperature. Instrument response function and weighted residuals of the fit is also included.

Singh et al., Mat. Chem. and Phys., 146, 136 – 140, 2014

VII. Other Corrections

1. Color effects in photomultiplier tube
2. Correction for color and geometric effects
3. Reference standards

VII. Other Corrections

1. Color effects and geometric effects

- Color effect: time dependent response of photocathode is wavelength dependent (PMTs can be selected for smallest possible color effect)
- Geometrical effect: time response of a PMT is also dependent on the area of the photocathode that is illuminated

VII. Other Corrections

2. Correction for color and geometric effects

- Adjust optics and / or PMT
- Establish deviation with standards of known lifetimes

VII. Other Corrections

3. Reference standards

For instrument evaluation, it is convenient to have a number of reference compounds with different lifetimes

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Fluorescence Lifetime Standards for Time and Frequency Domain Fluorescence Spectroscopy

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!! The environment (solvent) and temperature must be precisely controlled !!

Thanks for your attention!